

CLAIMS

We claim:

1. A method of selecting a zinc finger polypeptide that binds to a sequence of interest comprising at least two subsites, said method comprising the steps of:
  - 5 a) incubating position-sensitive primary libraries with target site constructs under conditions sufficient to form first binding complexes, wherein said primary libraries comprise zinc finger polypeptides having one variable finger and at least one anchor finger, and wherein the target site construct has one subsite with a sequence identical to a subsite of the sequence of interest, and one or more subsites with sequences to which the anchor  
10 finger(s) bind;
  - b) isolating pools comprising nucleic acid sequences encoding polypeptides, wherein said polypeptides comprise the first binding complexes;
  - c) recombining the pools to produce a secondary library;
  - 15 d) incubating the secondary library with the sequence of interest under conditions sufficient to form second binding complexes; and
  - e) isolating nucleic acid sequences encoding zinc finger polypeptides, wherein said polypeptides comprise the second binding complexes.
2. The method of claim 1, wherein the zinc finger polypeptide comprises at  
20 least two zinc fingers.
3. The method of claim 2, wherein the zinc finger polypeptide comprises three or more zinc fingers.
4. The method of claim 1, wherein the target site construct comprises the same number of base pairs as the sequence of interest.
- 25 5. The method of claim 1, wherein a subsite comprises 2-5 base pairs.
6. The method of claim 1, wherein the target site construct comprises two or more subsites.
7. The method of claim 1, wherein the target site construct comprises three or more subsites.
- 30 8. The method of claim 1, wherein one subsite of the target site construct has a sequence identical to the sequence of interest and the remaining subsite(s) in the target site construct have sequences that bind to the anchor finger(s).
9. The method of claim 8, wherein the remaining subsite(s) have sequences

selected from the group consisting of SEQ ID NO. 5 (GCC subsite 1), SEQ ID NO. 6 (GAA subsite 2) and SEQ ID NO. 7 (GCA subsite 3).

10. The method of claim 1, wherein the primary libraries comprise polypeptides having at least one anchor finger that is derived from a naturally occurring zinc  
5 finger polypeptide.
11. The method of claim 1, wherein the anchor finger(s) bind to subsites in the target site construct with low affinity and/or low specificity.
12. The method of claim 10, wherein the zinc finger polypeptide is selected from the group consisting of Zif268, tramtrack, GLI, YYI and TFIIIA.
- 10 13. The method of claim 12, wherein the zinc finger polypeptide is Zif268.
14. The method of claim 10, wherein the zinc finger polypeptide is a phage-selected derivative of Zif268.
15. The method of claim 14, wherein the phage-selected derivative of Zif268 comprises sequences selected from the group consisting of SEQ ID NO:2  
15 (DRSSLTR, finger 1), SEQ ID NO:3 (QGGNLVR, finger 2) and SEQ ID NO:4 (QAATLQR, finger 3).
16. The method of claim 1, wherein the variable finger is derived from a naturally occurring zinc finger polypeptide.
17. The method of claim 16, wherein the zinc finger polypeptide is selected from  
20 the group consisting of Zif268, tramtrack, YYI, GLI and TFIIIA.
18. The method of claim 17, wherein the zinc finger polypeptide is Zif268.
19. The method of claim 16, wherein the zinc finger polypeptide is a phage-selected derivative of Zif268.
20. The method of claim 19, wherein the phage-selected derivative of Zif268  
25 comprises sequences selected from the group consisting of SEQ ID NO:2 (DRSSLTR, finger 1), SEQ ID NO:3 (QGGNLVR, finger 2) and SEQ ID NO:4 (QAATLQR, finger 3) and combinations thereof.
21. The method of claim 1, wherein the variable zinc finger comprises six randomized amino acid residue positions located within, or just amino-terminal to  
30 the start of, the recognition alpha helix of the zinc finger.
22. The method of claim 21, wherein the randomized amino acid residue positions are -1, +1, +2, +3, +5 and +6, numbered with respect to the start of the recognition alpha helix of the zinc finger.

23. The method of claim 21, wherein between 16 to 20 amino acids are represented at each randomized position.
24. The method of claim 21, wherein between 16 to 19 amino acids are represented at each randomized residue position.
- 5 25. The method of claim 21, wherein 16 amino acids are represented at each randomized residue position.
26. The method of claim 1, wherein the primary libraries are expressed *in vitro*.
27. The method of claim 1, wherein the primary libraries are expressed in expression systems selected from the group consisting of eukaryotic, prokaryotic  
10 and viral expression systems.
28. The method of claim 27, wherein the primary libraries are expressed in bacteria.
29. The method of claim 1, wherein incubation of the primary libraries is performed *in vitro*.
- 15 30. The method of claim 1, wherein incubation of the primary libraries is performed within a prokaryotic or eukaryotic cell.
31. The method of claim 30, wherein the incubation is performed within a bacterial cell.
32. The method of claim 1, wherein the isolated pools of nucleic acid sequences  
20 are recombined to produce a secondary library by PCR-mediated recombination.
33. The method of claim 1, wherein the secondary library is expressed *in vitro*.
34. The method of claim 1, wherein the secondary library is expressed in an expression system selected from the group consisting of a eukaryotic, prokaryotic and viral expression system.
- 25 35. The method of claim 34, wherein the secondary library is expressed in bacteria.
36. The method of claim 1, wherein incubation of the secondary library with the sequence of interest is performed at high stringency to form a high-affinity binding complex.
- 30 37. The method of claim 1, wherein incubation of the secondary library is performed *in vitro*.
38. The method of claim 1, wherein incubation of the secondary library is performed within a prokaryotic or eukaryotic cell.

39. The method of claim 38, wherein the incubation of the secondary library is performed within a bacterial cell.
40. A method of regulating the expression of a gene comprising contacting a zinc finger polypeptide according to claim 1 with a sequence of interest in the gene to form a binding complex, such that expression of the gene is regulated.
41. A zinc finger polypeptide according to claim 1, wherein the zinc finger polypeptide is fused to one or more functional domains.
42. A method of regulating the expression of a gene comprising contacting a zinc finger polypeptide according to claim 41 with a sequence of interest in the gene.
43. A zinc finger polypeptide according to claim 41 wherein the functional domain is selected from the group comprising transcriptional activation domain, transcriptional repressor domain, transcriptional silencing domain, acetylase domain, de-acetylase domain, methylation domain, de-methylation domain, kinase domain, phosphatase domain, dimerization domain, multimerization domain, nuclear localization domain, nuclease domain, endonuclease domain, resolvase domain and integrase domain.
44. A zinc finger polypeptide according to claim 41 wherein the functional domain is an endonuclease domain.
45. A method of regulating the expression of a gene comprising contacting a zinc finger polypeptide according to claim 43 with a sequence of interest in the gene to form a binding complex, such that expression of the gene is regulated.
46. A method of altering the structure of a gene comprising contacting a zinc finger polypeptide according to claim 43 with a sequence of interest in the gene to form a binding complex, such that the structure of the gene is altered.
47. A method of cleaving a sequence of interest comprising contacting a zinc finger polypeptide according to claim 44 with the sequence of interest to form a binding complex, such that the sequence of interest is cleaved.
48. A method of selecting a chimeric zinc finger polypeptide that binds to a sequence interest comprising at least two subsites, said method comprising the steps of
- a) incubating position-sensitive primary libraries with target site constructs under conditions sufficient to form first binding complexes, wherein the position-sensitive primary libraries comprise zinc finger polypeptides having

one variable finger and at least one anchor finger, and wherein the target site constructs have one subsite with a sequence identical to a subsite of the sequence of interest, and one or more subsites with sequences to which the anchor finger(s) bind;

- 5       b) recombining said pools to produce a secondary library;
- c) incubating said secondary library with the sequence of interest under conditions sufficient to form second binding complexes;
- d) isolating nucleic acid sequences encoding multi-finger zinc finger polypeptides, wherein said polypeptides comprise the second binding
- 10       complexes, and
- e) fusing a nucleic acid sequence encoding a functional domain to the nucleic acid sequence encoding the multi-finger zinc finger polypeptides, to form a nucleic acid encoding a chimeric multi-finger zinc finger polypeptide

- 49.    The method of claim 48, wherein the zinc finger polypeptide comprises at
- 15    least two zinc fingers.
- 50.    The method of claim 49, wherein the zinc finger polypeptide comprises three or more zinc fingers.
- 51.    The method of claim 48, wherein the target site construct comprises the same number of base pairs as the sequence of interest.
- 20    52.    The method of claim 48, wherein a subsite comprises 2-5 base pairs.
- 53.    The method of claim 48, wherein the target site construct comprises two or more subsites.
- 54.    The method of claim 48, wherein the target site construct comprises three or more subsites.
- 25    55.    The method of claim 48, wherein one subsite of the target site construct has a sequence identical to the sequence of interest and the remaining subsite(s) in the target site construct have sequences that bind to the anchor finger(s).
- 56.    The method of claim 55, wherein the remaining subsite(s) have sequences selected from the group consisting of SEQ ID NO. 5 (GCC subsite 1), SEQ ID NO.
- 30    6 (GAA subsite 2) and SEQ ID NO. 7 (GCA subsite 3).
- 57.    The method of claim 48, wherein the primary libraries comprise polypeptides having at least one anchor finger that is derived from a naturally occurring zinc finger polypeptide.

58. The method of claim 48, wherein the anchor finger(s) bind to subsites in the target site construct with low affinity and/or low specificity.
59. The method of claim 57, wherein the zinc finger polypeptide is selected from the group consisting of Zif268, tramtrack, GLI, YYI and TFIIIA.
- 5 60. The method of claim 59, wherein the zinc finger polypeptide is Zif268.
61. The method of claim 57, wherein the zinc finger polypeptide is a phage-selected derivative of Zif268.
62. The method of claim 61, wherein the phage-selected derivative of Zif268 comprises sequences selected from the group consisting of SEQ ID NO:2  
10 (DRSSLTR, finger 1), SEQ ID NO:3 (QGGNLVR, finger 2) and SEQ ID NO:4 (QAATLQR, finger 3).
63. The method of claim 48, wherein the variable finger is derived from a naturally occurring zinc finger polypeptide.
64. The method of claim 63, wherein the zinc finger polypeptide is selected from  
15 the group consisting of Zif268, tramtrack, YYI, GLI and TFIIIA.
65. The method of claim 64, wherein the zinc finger polypeptide is Zif268.
66. The method of claim 63, wherein the zinc finger polypeptide is a phage-selected derivative of Zif268.
67. The method of claim 66, wherein the phage-selected derivative of Zif268  
20 comprises sequences selected from the group consisting of SEQ ID NO:2 (DRSSLTR, finger 1), SEQ ID NO:3 (QGGNLVR, finger 2) and SEQ ID NO:4 (QAATLQR, finger 3) and combinations thereof.
68. The method of claim 48, wherein the variable zinc finger comprises six randomized amino acid residue positions located within, or just amino-terminal to  
25 the start of, the recognition alpha helix of the zinc finger.
69. The method of claim 68, wherein the randomized amino acid residue positions are -1, +1, +2, +3, +5 and +6, numbered with respect to the start of the recognition alpha helix of the zinc finger.
70. The method of claim 68, wherein between 16 to 20 amino acids are  
30 represented at each randomized position.
71. The method of claim 68, wherein between 16 to 19 amino acids are represented at each randomized residue position.
72. The method of claim 68, wherein 16 amino acids are represented at each

randomized residue position.

73. The method of claim 48, wherein the primary libraries are expressed *in vitro*.

74. The method of claim 48, wherein the primary libraries are expressed in expression systems selected from the group consisting of eukaryotic, prokaryotic  
5 and viral expression systems.

75. The method of claim 74, wherein the primary libraries are expressed in bacteria.

76. The method of claim 48, wherein incubation of the primary libraries is performed *in vitro*.

10 77. The method of claim 48, wherein incubation of the primary libraries is performed within a prokaryotic or eukaryotic cell.

78. The method of claim 77, wherein the incubation is performed within a bacterial cell.

79. The method of claim 48, wherein the isolated pools of nucleic acid sequences  
15 are recombined to produce a secondary library by PCR-mediated recombination.

80. The method of claim 48, wherein the secondary library is expressed *in vitro*.

81. The method of claim 48, wherein the secondary library is expressed in an expression system selected from the group consisting of a eukaryotic, prokaryotic and viral expression system.

20 82. The method of claim 81, wherein the secondary library is expressed in bacteria.

83. The method of claim 48, wherein incubation of the secondary library with the sequence of interest is performed at high stringency to form a high-affinity binding complex.

25 84. The method of claim 48, wherein incubation of the secondary library is performed *in vitro*.

85. The method of claim 48, wherein incubation of the secondary library is performed within a prokaryotic or eukaryotic cell.

86. The method of claim 85, wherein the incubation of the secondary library is  
30 performed within a bacterial cell.

87. A method of regulating the expression of a gene comprising contacting a zinc finger polypeptide according to claim 48 with a sequence of interest in the gene to form a binding complex, such that expression of the gene is regulated.

88. A zinc finger polypeptide according to claim 48, wherein the zinc finger polypeptide is fused to one or more functional domains.
89. A method of regulating the expression of a gene comprising contacting a zinc finger polypeptide according to claim 48 with a sequence of interest in the gene.
- 5 90. A zinc finger polypeptide according to claim 88 wherein the functional domain is selected from the group comprising transcriptional activation domain, transcriptional repressor domain, transcriptional silencing domain, acetylase domain, de-acetylase domain, methylation domain, de-methylation domain, kinase domain, phosphatase domain, dimerization domain, multimerization domain, nuclear
- 10 localization domain, nuclease domain, endonuclease domain, resolvase domain and integrase domain.
91. A zinc finger polypeptide according to claim 88 wherein the functional domain is an endonuclease domain.
92. A method of regulating the expression of a gene comprising contacting a
- 15 zinc finger polypeptide according to claim 89 with a sequence of interest in the gene to form a binding complex, such that expression of the gene is regulated.
93. A method of altering the structure of a gene comprising contacting a zinc finger polypeptide according to claim 90 with a sequence of interest in the gene to form a binding complex, such that the structure of the gene is altered.
- 20 94. A method of cleaving a sequence of interest comprising contacting a zinc finger polypeptide according to claim 91 with the sequence of interest to form a binding complex, such that the sequence of interest is cleaved.
95. A position-sensitive primary library comprising zinc finger polypeptides having one variable finger and at least one anchor finger, wherein the position of the
- 25 variable finger is the same as the position of the corresponding zinc finger in a multi-finger zinc finger polypeptide.